

# Decline in Self-Renewal Factors Contributes to Aging of the Stem Cell Niche in the *Drosophila* Testis

Monica Boyle,<sup>1,3</sup> Chihunt Wong,<sup>1,2,3</sup> Michael Rocha,<sup>1</sup> and D. Leanne Jones<sup>1,2,\*</sup>

<sup>1</sup>Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

<sup>2</sup>Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92037, USA

<sup>3</sup>These authors contributed equally to this work.

\*Correspondence: [ljones@salk.edu](mailto:ljones@salk.edu)

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## SUMMARY

Aging is characterized by compromised organ and tissue function. A decrease in stem cell number and/or activity could lead to the aging-related decline in tissue homeostasis. We have analyzed how the process of aging affects germ line stem cell (GSC) behavior in the *Drosophila* testis and report that significant changes within the stem cell microenvironment, or niche, occur that contribute to a decline in stem cell number over time. Specifically, somatic niche cells in testes from older males display reduced expression of the cell adhesion molecule DE-cadherin and a key self-renewal signal *unpaired* (*upd*). Loss of *upd* correlates with an overall decrease in stem cells residing within the niche. Conversely, forced expression of *upd* within niche cells maintains GSCs in older males. Therefore, our data indicate that age-related changes within stem cell niches may be a significant contributing factor to reduced tissue homeostasis and regeneration in older individuals.

## INTRODUCTION

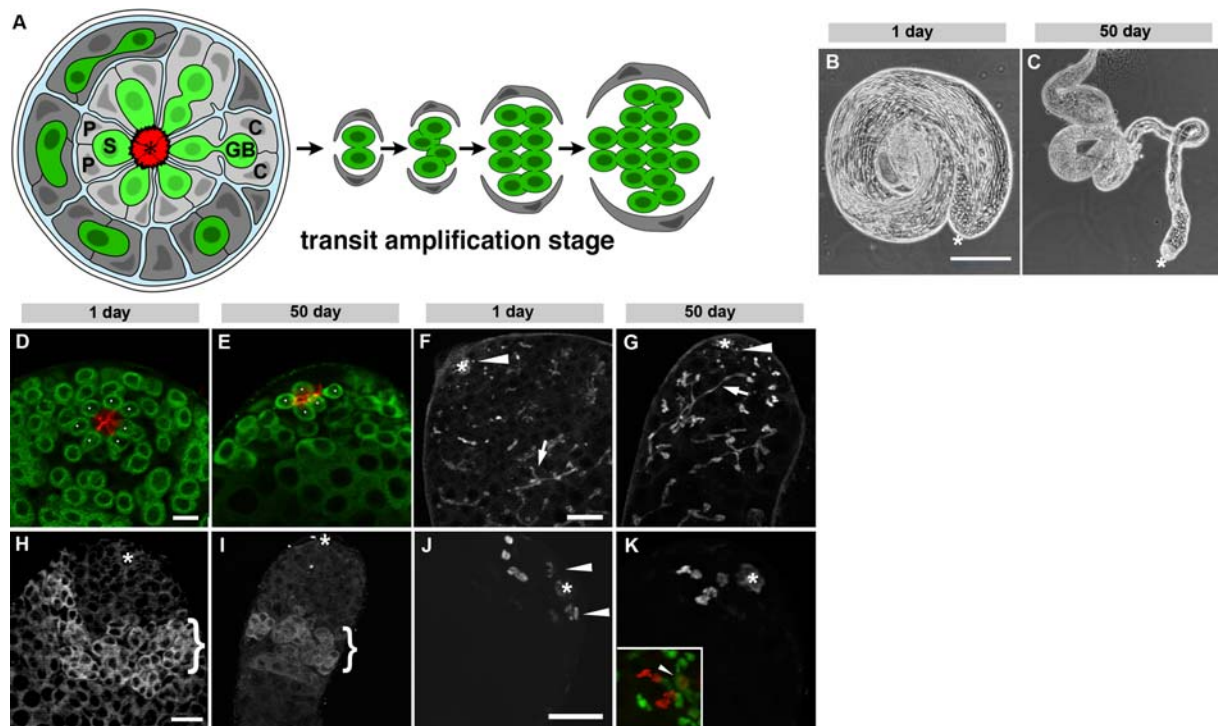
Adult stem cells, also known as tissue stem cells, support tissue homeostasis and repair throughout the life of an individual. However, maintenance and regeneration of tissues such as skin, liver, blood, and muscle decrease dramatically with age. Cell-intrinsic changes have been proposed to play a role in the observed decrease in stem cell function (reviewed in [Rando, 2006](#)). For example, increased expression of the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> correlates with aging of hematopoietic stem cells (HSCs) and neural progenitor cells, possibly contributing to an age-related decline in HSC repopulating activity and neurogenesis ([Janzen et al., 2006](#); [Molofsky et al., 2006](#)). Studies have also demonstrated that cell-extrinsic changes contribute to a decline in the ability of

aged stem cells to repair damaged tissues ([Conboy et al., 2005](#)).

Many stem cell populations lose the capacity for self-renewal when removed from the stem cell niche, suggesting that the local environment plays a major role in controlling stem cell fate ([Morrison et al., 1997](#); [Schofield, 1978](#)). Therefore, changes to the niche could contribute to a decline in stem cell number and activity during aging. Transplantation studies suggest that the age-related decline in spermatogenesis observed in 2-year-old mice is due to aging of the niche rather than intrinsic changes within spermatogonial stem cells ([Ryu et al., 2006](#); [Zhang et al., 2006](#)). However, the precise molecular changes within the stem cell environment have been difficult to assess due to the lack of identified niche components in many stem cell systems. Consequently, mechanisms underlying the age-related decline in stem cell activity in many tissues are not well understood.

*Drosophila* is a well-established model for studying organismal aging and functional senescence as well as mechanisms that regulate stem cell behavior ([Grotewiel et al., 2005](#); [Helfand and Rogina, 2003](#); [Yamashita et al., 2005](#)). *Drosophila* spermatogenesis is maintained by a population of GSCs that lie at the tip of the testis surrounding a cluster of somatic cells called the apical hub ([Figure 1A](#)) ([Hardy et al., 1979](#)). Hub cells secrete the ligand Unpaired (Upd), which activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction pathway in adjacent GSCs to specify stem cell self-renewal ([Harrison et al., 1998](#); [Kiger et al., 2001](#); [Tulina and Matunis, 2001](#)). Therefore, hub cells are an essential component of the stem cell niche in the *Drosophila* testis ([Kiger et al., 2001](#); [Tulina and Matunis, 2001](#)).

We have analyzed the effects of aging on stem cells and the niche in the *Drosophila* testis. Here, we show that levels of *upd* decline with age and that this loss correlates with a reduction in GSCs residing within the niche ([Wallenfang et al., 2006](#)). Conversely, forced expression of *upd* within hub cells maintains GSCs in older males. Therefore, our data indicate that a decline in self-renewal factors is one mechanism that contributes to decreased niche function with age, leading to reduced tissue homeostasis and impaired regeneration in older individuals.



**Figure 1. Decreased Spermatogenesis and GSC Loss with Age**

(A) The apical tip of the *Drosophila* testis illustrating the early stages of spermatogenesis (modified from Hardy et al., 1979). GSCs (S) and somatic cyst progenitor cells, CPCs (P), surround the hub (red). Gonialblasts (GB) are enveloped by cyst cells (C) and undergo four mitotic divisions to create a cyst of 16 spermatogonia.

(B and C) Phase contrast images of testes from (B) 1- and (C) 50-day-old *yw;nanosGAL4:VP16,UAS- $\alpha$ -tubulin:gfp* (Tubulin:GFP) adults. Asterisks mark the testis tip. Note decreased size at 50 days.

(D and E) Staining for the germ cell antigen Vasa (green) and the cell surface protein Fasciclin III (FasIII, red) in the hub in (D) 1- and (E) 50-day-old males. GSCs are marked by a white dot, with 7 GSCs in (D) and 5 GSCs in (E).

(F and G) FasIII (\*) staining and  $\alpha$ -spectrin, which marks spherical fusomes in GSCs and GB (arrowheads) and branched fusomes in spermatogonia (arrows). (F) One section through a 1-day and (G) projection of 5 through a 50-day-old testis, each with fusomes in germ cells near the hub (arrowheads). Note highly branched fusomes, representing 8–16 cell spermatogonial cysts, closer to the tip in (G).

(H and I) Staining for BamC, a 4–8 cell spermatogonial cyst marker in (H) 1- and (I) 50-day-old Tubulin:GFP testes. Note reduced BamC region (brackets) in (I).

(J and K) Staining for phospho-histone H3 (pHH3) to label mitotic cells in (J) 1- and (K) 50-day-old testes. Note dividing GSCs (arrowheads) and two cells of a 4 cell cyst in (J) and a 2 cell cyst in (K). Inset shows a dividing 2 cell cyst labeled by pHH3 (red) and a dividing cell (arrowhead) positive for Traffic Jam (TJ, green), a marker of CPCs and early cyst cells. Dividing GSCs were counted as pHH3<sup>+</sup>, TJ<sup>−</sup> cells adjacent to the hub. Scale bars: (B), 250  $\mu$ M; (D), 10  $\mu$ M; (F), H, J, 20  $\mu$ M. Testes are from OregonR flies and all images are single sections unless otherwise indicated. Asterisks mark hub.

## RESULTS

### Age-Related Decline in GSCs

Asymmetric division of GSCs begins in the testis by late embryogenesis and maintains spermatogenesis throughout larval development and adulthood. Upon GSC division, the daughter cell that maintains contact with the hub retains stem cell identity, while the displaced daughter cell initiates differentiation as a gonialblast (Figure 1A; Hardy et al., 1979). Gonialblasts undergo four mitotic divisions to generate a cyst of 16 spermatogonia that differentiate into spermatocytes and, eventually, 64 mature sperm.

The mean lifespan of *D. melanogaster* is approximately 40 days (Helfand and Rogina, 2003). Analysis of testes from wild-type 30- and 50-day-old aged males revealed

a marked decrease in spermatogenesis: testes from aged males were thinner than testes from young (1- to 2-day-old) males and contained fewer differentiating germ cells (Figures 1B and 1C). One explanation for the observed decrease in spermatogenesis with age is loss of GSCs. Quantification of GSC number in testes of 1-, 30-, and 50-day-old males revealed a significant decrease from an average of 8.3 GSCs/testis in 1-day-old to 6.11 GSCs/testis in 30-day-old and 5.1 GSCs/testis in 50-day-old OregonR males ( $p < 0.05$ ; Figures 1D and 1E; Table S1 and Figures S1A and S1B in the Supplemental Data available with this article online). These data are consistent with studies reporting a 25% decrease in GSCs in 35-day-old males (Wallenfang et al., 2006).

Although the number of GSCs decreased in older males, the remaining GSCs continued to express

hallmarks of stem cells. GSCs contain a spherical, cytoplasmic structure called a fusome, which becomes branched as germ cells differentiate as interconnected spermatogonia (Figure 1F; Lin et al., 1994). GSCs in aged testes contained spherical fusomes; however, highly branched fusomes were found closer to the tip of the testis when compared to testes from 1-day-old males, indicating fewer differentiating spermatogonial cysts (Figures 1F and 1G). Furthermore, single germ cells adjacent to the hub of older males showed no overt signs of differentiating prematurely (Figures 1H and 1I). The *bag of marbles* (*bam*) gene is expressed in differentiating spermatogonia within 4–16 cell cysts and is required for limiting the number of mitotic divisions (Gönczy et al., 1997; McKearin and Ohlstein, 1995). Testes stained with antibodies for the cytoplasmic pool of Bam protein (BamC) indicated that dividing spermatogonia within older testes initiate differentiation similar to spermatogonia in 1-day-old males.

#### Decline in GSC Divisions in Testes from Aging Males

To determine whether GSCs in testes from older males continue to divide, antibodies to phospho-histone H3, a marker of mitosis, were used to stain testes at 1 and 50 days. At 1 day, an average of 0.9 dividing GSCs/testis were observed ( $n = 20$ ), whereas an average of 0.4 dividing GSCs/testis were observed at 50 days ( $n = 50$ ; Figures 1J and 1K). The decrease in dividing GSCs was significant ( $p < 0.05$ ) and similar to the 40% reduction in total GSCs surrounding the hub (Table S1).

Staining with another marker of cell-cycle progression verified that GSCs present in testes from old males continue to divide, albeit less often than GSCs in testes from young males. BrdU labeling, which marks cells in S phase, showed a decrease in the average number of dividing GSCs in aged OregonR testes: 1.6 BrdU<sup>+</sup> GSCs/testis in 1-day-old males ( $n = 51$ ) in contrast to an average of 0.8 BrdU<sup>+</sup> GSCs/testis in 50-day-old males ( $n = 59$ ). These data revealed a significant drop in GSC proliferation rate in 50-day-old males when compared to 1-day-old males (S phase index = average number of BrdU<sup>+</sup> GSCs/average number of GSCs,  $p < 0.05$ ; Table S2) and are consistent with studies reporting a decline in GSC activity at earlier times (Hardy et al., 1979; Tran et al., 2000; Wallenfang et al., 2006). We conclude that fewer GSCs, in combination with decreased GSC divisions, contribute to fewer differentiating progenitor cells (spermatogonia and spermatocytes), ultimately leading to the observed decrease in spermatogenesis in aged males.

The average number of GSCs expressing high levels of cyclin E, a regulator of progression through the G1 phase of the cell cycle, increased in testes from older males (Figure S2). Cyclin E levels varied, with a distribution of high and low levels in GSCs from 1-day-old males ( $n = 20$ ). However, testes from 50-day-old males showed more GSCs expressing high levels of cyclin E (Figure S2C). High cyclin E levels are consistent with an arrest in or extension of G1 and may explain the subtle decrease in the percentage of cells progressing through S phase (Hatfield

et al., 2005; Table S2). An age-related accumulation of Dacapo, the *Drosophila* homolog of the CIP/KIP family of cyclin-dependent kinase inhibitors, was not apparent in GSCs from older males (data not shown; de Nooij et al., 1996; Lane et al., 1996). However, upregulation of *dacapo* expression or protein is not always coupled to cell-cycle arrest (Meyer et al., 2002).

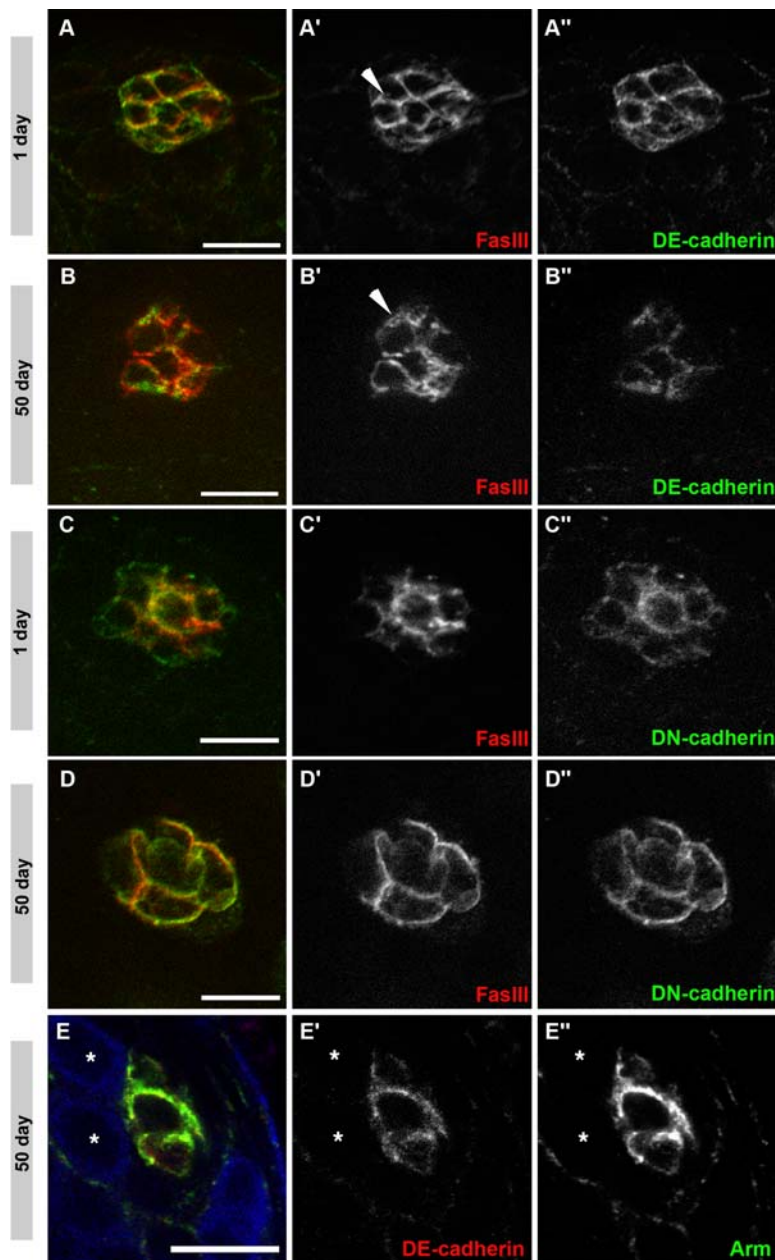
A second stem cell population, the somatic cyst progenitor cells (CPCs), is located at the tip of the testis (Figure 1A; Hardy et al., 1979). Self-renewing CPCs contact both GSCs and hub cells and give rise to cyst cells that enclose the differentiating gonialblast. CPCs are distinguished from other somatic cells in the testis based on their mitotic activity and position adjacent to the hub (Hardy et al., 1979). Dividing somatic cells were detected adjacent to the hub in both 1- and 50-day-old males using both pHH3 staining and BrdU incorporation (Figure 1K; Table S2). However, an assessment of any aging-related decline in CPC number or division rate was hindered by a lack of specific markers to distinguish CPCs from differentiating cyst cells at early stages.

#### The Apical Hub Remains Intact in Testes from Aged Males

We next examined whether there were detectable changes to a key component of the stem cell niche in the testis: the apical hub. The hub consists of approximately 8–16 cells that express high levels of cell adhesion molecules such as Fasciclin III (FasIII; Gönczy et al., 1997) and the *Drosophila* homologs of E-cadherin, (DE-cadherin or DE-cad) and neural cadherin, DN-cadherin (DN-cad; Le Bras and Van Doren, 2006).

The hub was still detectable in testes from aged males as assayed by four markers (Figure S3). Quantification of hub cell number using DN-cad revealed a small but significant drop in the total number of cells from  $11.4 \pm 0.48$  at 1 day ( $n = 23$ ) to  $9.3 \pm 0.35$  at 50 days ( $n = 23$ ;  $p < 0.05$ ), and only 2.0% of testes (3/147) showed complete loss of FasIII staining in OregonR males ( $\pm$  = Standard Error; Figures S3A and S3B). The small decline in hub cell number is consistent with, but less dramatic than, previous findings (Wallenfang et al., 2006). Although the overall diameter of the hub was normal in the majority of testes from older males, an increase in hub diameter from 10–15  $\mu\text{m}$  to larger than 20  $\mu\text{m}$  was observed in 5.4% (8/147) of the testes examined, as assayed by FasIII staining (Figures S3D–S3F). An expansion of somatic cells in testes and ovaries often accompanies loss of germ cells, one example being an expansion of FasIII expressing cells in agametic gonads from young flies (Gönczy and DiNardo, 1996; Margolis and Spradling, 1995).

A consistent decrease in DE-cad levels was also observed within the hub in testes from aged males compared to 1-day-old testes (Figures 2A' and B'). In contrast, significant changes in DN-cad levels were not observed (Figures 2C and 2D). The *Drosophila* homolog of  $\beta$ -Catenin, Armadillo, was still expressed and colocalized with DE-cad, suggesting that the remaining DE-cad was capable of binding to core adherens junctions



**Figure 2. Aging-Related Changes to the Apical Hub**

(A–D) View of the hub stained with FasIII (red) and the cell adhesion molecules DE-cadherin (DE-cad, green, [A and B]) or DN-cadherin (DN-cad, green, [C and D]) in 1- (A and C) and 50-day old (B and D) testes. DE-cad expression level is decreased in 50-day-old testes compared to 1 day (compare [A'] to [B']). In contrast, DN-cad levels are similar at 1 and 50 days (compare [C'] to [D']). Note change in FasIII localization: at 1 day, FasIII is concentrated along hub cell/hub cell junctions (arrowhead in [A']), with little or no staining along the hub periphery. In aged testes, FasIII is often present at the periphery (arrowhead in [B']). (E) View of the hub stained with DE-cad (red, [E']) and Armadillo (green, [E'']) in 50-day old testis. Germ cells stained for Vasa (blue). Asterisks mark GSCs. Scale bars, 10  $\mu$ M.

components (Figure 2E). As DE-cad is required for stem cell maintenance in the *Drosophila* gonad, a decrease in expression in either hub cells or GSCs could contribute to loss of GSCs with age (Song and Xie, 2002; Song et al., 2002; J. Voog and D.L.J., unpublished data).

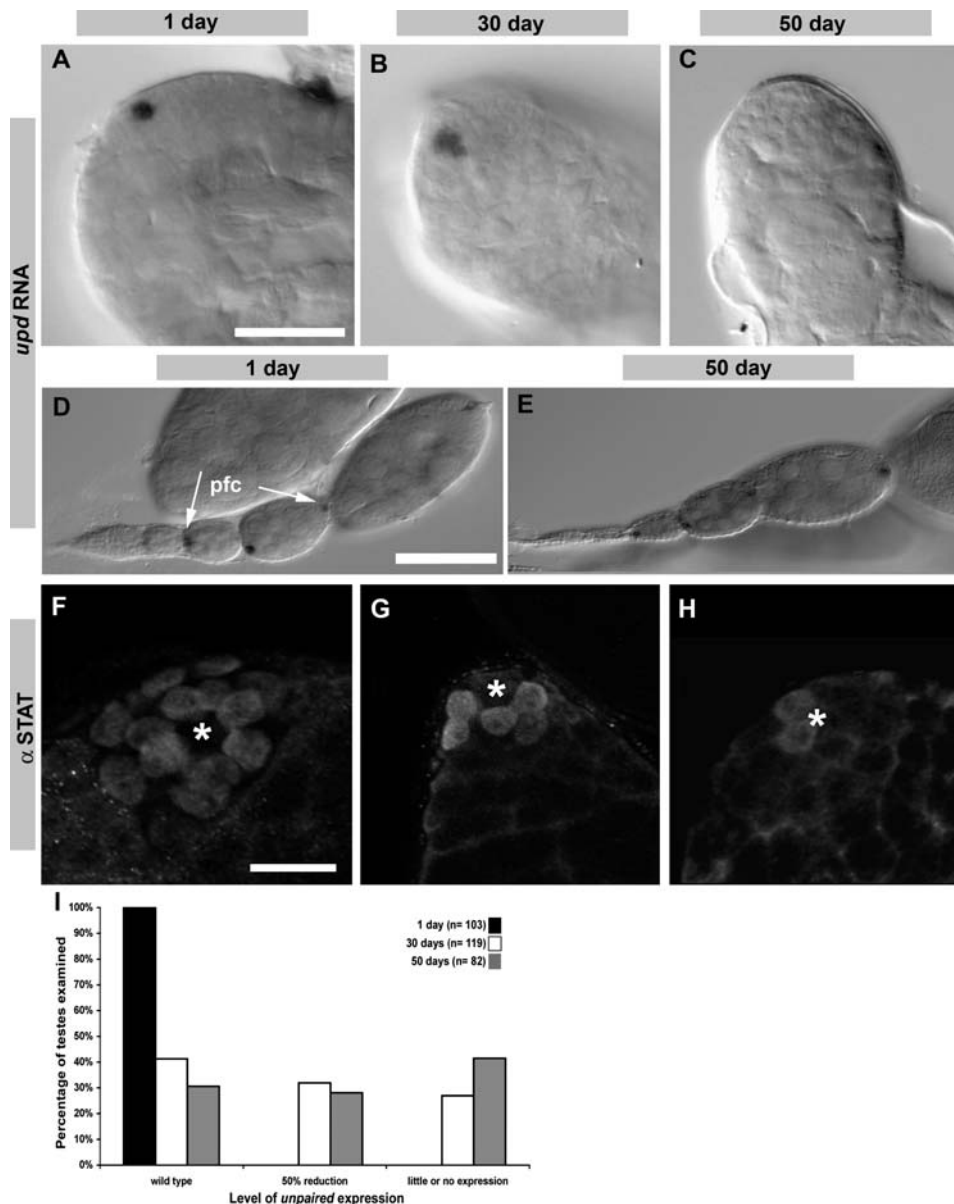
#### Age-Related Decline in Stem Cell Niche Function

Previous analyses demonstrated that *upd* is sufficient for specifying GSC self-renewal in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001). Therefore, we assayed *upd* expression within hub cells in testes from aging males. Analysis of *upd* mRNA expression showed a progressive loss in hub cells in testes from aged males (Figures 3A–3C). While wild-type levels of *upd* expression

in the hub were detected in 99% (103/104) of 1-day-old testes, only 30%–40% of testes from 30-day- (49/119) and 50-day-old (25/82) males displayed wild-type or nearly wild-type levels of *upd* (Figures 3A–3C and Figure 3I; Figure S4F). In contrast, expression of the transcription factor *escargot*, which is normally detected in hub cells and surrounding stem cells, was still evident in hub cells in testes from 30- and 50-day-old flies, despite a significant decrease in the surrounding stem cells (Figure S4).

Declining *upd* expression in the testis during aging could be specific to the male GSC niche or reflect a general decline in *upd* expression levels with age. To distinguish between these possibilities, we examined *upd* expression





**Figure 3. Aging-Related Changes in JAK-STAT Signaling**

(A–C) RNA in situ analysis for *upd*, which encodes a ligand of the JAK-STAT pathway, in the hub in (A) 1-, (B) 30-, and (C) 50-day-old testes. (B) shows a 50% decrease compared to wild-type, with complete loss of expression shown in (C).

(D and E) *upd* mRNA expression in polar follicle cells (pfc, arrows) in ovarioles from (D) 1- and (E) 50-day-old females.

(F–H) Staining for Stat92E, a target of the JAK-STAT pathway, in (F) 1-, (G) 30-, and (H) 50-day-old testes showing fewer Stat92E positive (Stat<sup>+</sup>) cells with age. Asterisks mark hub. (I) Distribution of *upd* expression levels in testes from 1-, 30-, and 50-day-old flies. Numbers are combined from four independent experiments. Scale bars: (A) and (F), 20  $\mu$ M; (D), 50  $\mu$ M.

in ovaries from aging females. Expression of *upd* in distinct subsets of cells within the ovary activates JAK-STAT signaling to specify and guide migration of specialized somatic cells (McGregor et al., 2002; Silver and Montell, 2001). In a majority of ovarioles from 50-day-old females (114/116), *upd* expression was still detectable (Figures 3D and 3E), indicating that aging-related changes in *upd* levels do not occur in all tissues in which *upd* is expressed.

Decreased *upd* expression in hub cells suggested that JAK-STAT signaling might be compromised within the niche. Stat92E is known to regulate its own expression, leading to an increase in Stat92E protein. Therefore, an overall increase in Stat92E protein in combination with Stat92E nuclear localization can be used to assay JAK-STAT pathway activation (Wawersik et al., 2005). The average number of Stat92E positive (Stat<sup>+</sup>) germ cells was 15 in 1-day-old ( $n = 40$ ), 9.8 in 30-day-old ( $n = 50$ ),

and 6.9 in 50-day-old ( $n = 51$ ) OregonR testes, which corresponded to 7.8 Stat<sup>+</sup> GSCs at 1 day ( $n = 20$ ), 5.5 Stat<sup>+</sup> GSCs at 30 days ( $n = 23$ ), and 4.7 Stat<sup>+</sup> GSCs at 50 days ( $n = 22$ ; Figures 3F–3H; Tables S3 and S4). These data demonstrate a significant decrease in early germ cells responding to JAK-STAT signaling in aged males ( $p < 0.05$ ).

### Constitutive Expression of *upd* in Hub Cells Delays Loss of GSCs

To determine whether sustained *upd* expression could rescue the aging-related decline in GSCs, flies in which *upd* was constitutively expressed in hub cells were aged (Brand et al., 1994). A modest, yet significant increase in GSCs was observed in testes from these males when compared to age-matched controls (Figures 4A and 4B; Table S3). Furthermore, *upd* expression was now easily detectable in the majority of 50-day old testes (Figures S4D–S4F). When another transgenic line was used to express *upd* in hub cells, GSC loss was essentially blocked in aging males. The average number of GSCs in testes from 30-day old males remained constant at 7.4 GSCs/testis ( $n = 68$ ), corresponding to an average of 6.0 Stat<sup>+</sup> GSCs/testis ( $n = 22$ ; Figure 4; Tables S3 and S4). In contrast, controls showed the expected 20%–25% decline to 6.4 GSCs/testis ( $n = 38$ ), which corresponded to 4.6 Stat<sup>+</sup> GSCs/testis ( $n = 27$ ,  $p < 0.05$ ; Figure 4, Tables S3 and S4). Therefore, not only were GSCs maintained upon constitutive expression of *upd*, but JAK-STAT activation was also preserved, suggesting that the decrease in *upd* expression in hub cells is an important contributing factor to the decline in GSCs with age.

Despite the maintenance of JAK-STAT responsive GSCs, testes in which *upd* was constitutively expressed in hub cells showed a decline in the total number of germ cells responding to the pathway as few or no Stat<sup>+</sup> gonialblasts were observed (Figures 4E and 4F; compare to Figure 2F; Table S4). One explanation for this could be a decline in the proliferation rate of GSCs, leading to a decrease in the pool of early germ cells available to respond to JAK-STAT signaling. GSC proliferation rates in testes constitutively expressing *upd* in the hub were assayed using BrdU incorporation, which revealed a significant decrease in GSC proliferation between 1 and 30 days (Figure 4J). However, when compared to 30-day-old controls, a further decrease in the rate of GSC proliferation was observed (Figure 4J). Consistent with this observation, cyclin E levels in GSCs in testes in which *upd* expression was maintained in the hub were also considerably higher than in 30 day controls (Figure S2). Therefore, a decrease in the rate of GSC proliferation upon constitutive expression of *upd* in hub cells could provide one explanation for the decline in early germ cells responding to the JAK-STAT pathway.

Consistent with a decline in GSC proliferation and loss of Stat<sup>+</sup> gonialblasts, spermatogenesis was not restored in testes in which *upd* is constitutively expressed in hub cells. In fact, testes from 30-day-old males were thinner than controls and resembled testes from considerably older males due to a dramatic decrease in developing germ cells

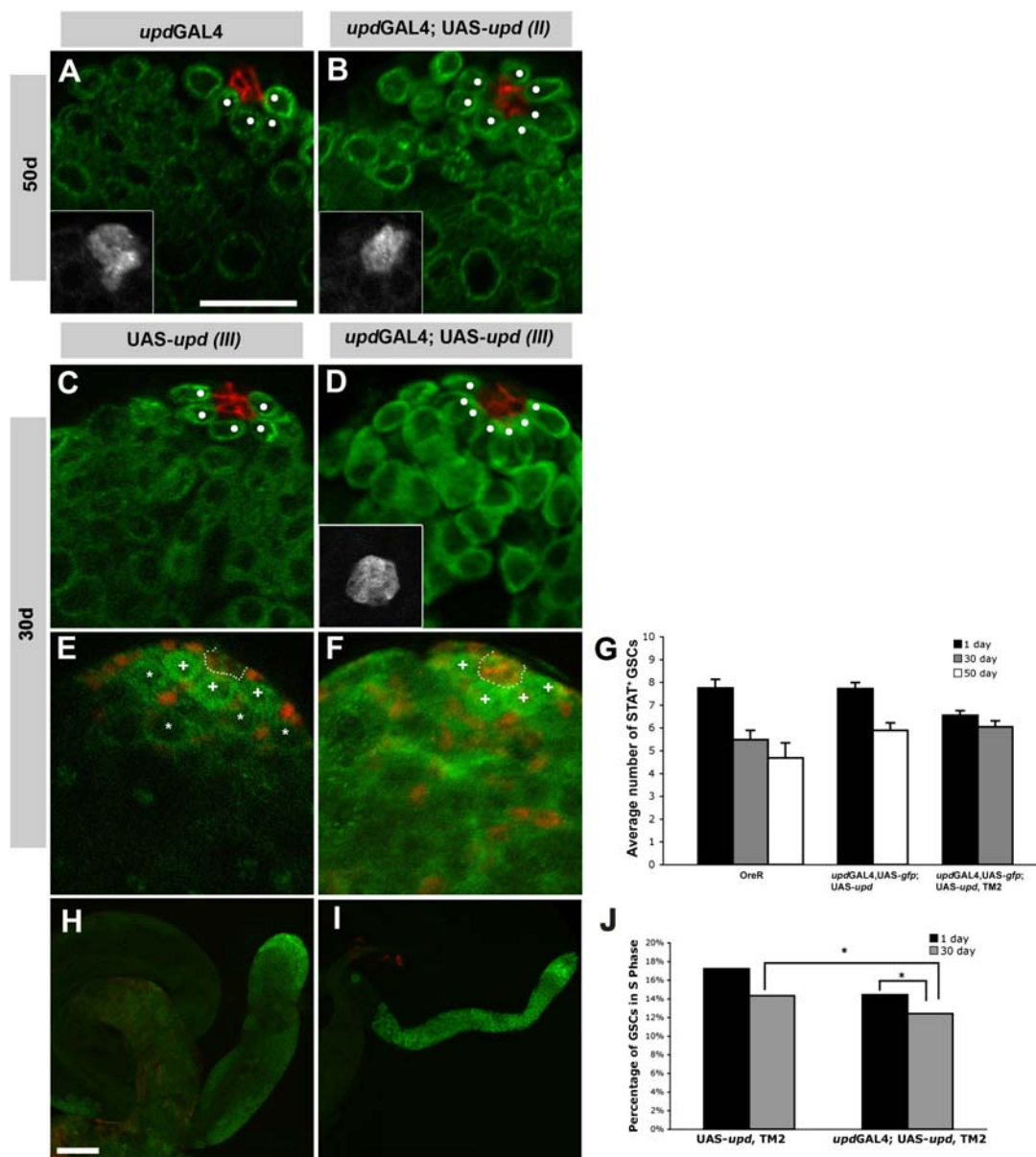
found within the testis lumen (Figures 4H and 4I; compare to Figures 1A and 1B). Furthermore, these flies did not live much beyond 30 days, preventing any analysis at later time points. Based on the accelerated decline in GSC proliferation and spermatogenesis and shortened life span, we hypothesize that flies overexpressing *upd* at higher than physiological levels, but within the normal domain, may be dying due to acceleration of the aging process.

### DISCUSSION

Our data indicate that aging results in molecular changes within a stem cell niche, including a decrease in expression of a key self-renewal factor, *upd*. We propose that the reduction in *upd* reflects a change in niche function, which contributes to loss of GSCs over time. Ultimately, fewer GSCs within the niche leads to a subsequent decrease in the number of progenitor cells that generate mature sperm, resulting in an overall decrease in spermatogenesis in aged males.

In addition to the decline in *upd* expression, we observed a consistent decrease in DE-cad levels within the hub in testes from aged males (Figure 2). As localized adherens junctions have been demonstrated to be required for holding stem cells within the niche, close to self-renewal signals, a decrease in DE-cad expression in either hub cells or GSCs could contribute to GSC loss in aged testes (Song and Xie, 2002; Song et al., 2002). Experiments to examine DE-cad function within hub cells and GSCs during aging are ongoing. In contrast to *upd*, our data to date suggest that overexpressing DE-cad in the hub alone does not result in GSC maintenance in older males (M.B. and D.L.J., unpublished data). However, a considerable decrease in *upd* expression in niche cells was observed at 30 days, which preceded any detectable change in DE-cad expression; therefore, the decrease in *upd* expression is one of the first molecular hallmarks of aging within the niche.

Despite a net decrease in stem cells, the actual decline in GSCs during aging is less than one might predict. A recent study of the dynamics of GSC behavior in 30-day-old males revealed the half-life of GSCs to be 14 days (Wallenfang et al., 2006). However, more than half (65%) of GSCs remain at 50 days, suggesting that mechanisms are in place to maintain a stem cell pool within the niche (Table S1; Wallenfang et al., 2006). Similar findings have been made for GSC maintenance in the *Drosophila* ovary (Margolis and Spradling, 1995; Xie and Spradling, 1998, 2000). Replacement of lost stem cells could occur through symmetric divisions of remaining GSCs, as was shown for female GSCs in *Drosophila* (Xie and Spradling, 2000). Another possibility is that dedifferentiation of spermatogonia into functional GSCs maintains GSCs in older males (Brawley and Matunis, 2004). An extension of the half-life of male GSCs to longer than 14 days or a slowed division rate (>32 hr) could also delay stem cell exhaustion. The observed increase in cyclin E levels in aged GSCs may indicate an arrest or significant delay in the G1 phase of the cell cycle (Figure S2).



**Figure 4. Constitutive Expression of *upd* Delays Loss of GSCs During Aging**

The GAL4-UAS system, which allows temporal and tissue specific gene expression, was used to express *upd* in hub cells.

(A and B) Testes stained for Vasa (green) and FasIII (red) from 50-day-old (A) *updGAL4,UAS-gfp* and (B) *updGAL4,UAS-gfp;UAS-upd* males.

(C and D) Vasa (green) and FasIII (red) staining in 30-day-old (C) *UAS-upd, TM2/+* and (D) *updGAL4,UAS-gfp; UAS-upd, TM2/+* males. Insets show GFP staining in hub cells. Dots mark GSCs.

(E and F) Stat92E<sup>+</sup> cells in testes overexpressing *upd* in hub cells. Testes stained for Stat92E (green) and TJ (red) in 30-day old (E) *UAS-upd, TM2/+* and (F) *updGAL4,UAS-gfp; UAS-upd, TM2/+* males. + marks Stat<sup>+</sup> GSCs. Asterisks mark Stat<sup>+</sup> gonialblasts, GB. (E) 3 of 6 total GSCs are evident in this section, while 4 of 7 GBs are visible. (F) 4 of 7 total GSCs and 0 of 2 total GBs are evident in this section. Note activation of JAK-STAT pathway in the hub (outline) as visualized by Stat92E staining (compare with Figures 2F–2H).

(G) Maintenance of Stat<sup>+</sup> GSCs upon constitutive expression of *upd* in both *updGAL4,UAS-gfp;UAS-upd* and *updGAL4,UAS-gfp;UAS-upd, TM2/+* males as compared to OregonR males during aging.

(H and I) Low magnification views of testes from 30-day-old (H) *UAS-upd, TM2/+* and (I) *updGAL4,UAS-gfp;UAS-upd, TM2/+* males.

(J) Graph representing drop in proliferation rate upon constitutive expression of *upd* from 14.4% in 1-day-old to 12.4% in 30-day-old *updGAL4,UAS-gfp; UAS-upd, TM2/+* males. Asterisks: statistically significant ( $p < 0.05$ ). Genotypes: (A) *updGAL4,UAS-gfp(Y); +/+; +/+*. (B) *updGAL4, UAS-gfp(Y); UAS-upd/+ (II); +/+*. (C, E, and G) *+/Y; +/+; UAS-upd, TM2/+ (III)* (D, F, and H) *updGAL4, UAS-gfp(Y); +/+; UAS-upd, TM2/+ (III)*. Scale bars: (A)–(F), 20  $\mu$ M; (H) and (I), 100  $\mu$ M.

Previous studies have shown that JAK-STAT signaling is necessary and sufficient to specify stem cell self-renewal in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001). Here we show that constitutive expression of *upd* within the hub maintains GSCs during aging. In addition to specifying self-renewal, *Upd* may also facilitate, either directly or indirectly, the ability of existing germ cells to replace lost GSCs, thereby maintaining an adequate number of GSCs within the niche. We predict that as *upd* levels within the hub diminish during aging, mechanisms that serve to maintain GSCs are compromised, leading to loss of GSCs. Mechanisms that replace lost stem cells to maintain an active stem cell pool must be in constant competition with cell autonomous, local, and systemic changes that occur during aging (Figure S5); once stem cell replacement becomes less efficient, the overall balance tips toward loss of active stem cells.

Clearly, multiple factors are likely to influence GSC number and activity over time. Similar to what has been proposed in mammalian systems, cell autonomous changes may occur that either block cell-cycle progression or prevent stem cells from responding to self-renewal cues (Janzen et al., 2006; Molofsky et al., 2006; Ryu et al., 2006; Figure S5). Variation in systemic factors, such as *Drosophila* insulin-like peptides (dILPs), ecdysone, or juvenile hormone (JH), could also act directly or indirectly to affect stem cell activity during aging (Conboy et al., 2005; LaFever and Drummond-Barbosa, 2005). Understanding how local, systemic, and cell autonomous changes are integrated to elicit changes in stem cell behavior that occur over time presents an exciting challenge (reviewed in Rando, 2006).

Our data suggest that aging results in loss of functional stem cell niches. Compromised niche function over time may lead to the selection of stem cells that acquire the ability to self-renew independently of the niche and/or progenitor cells that acquire self-renewal capabilities (Li and Neaves, 2006). Such cells could be the precursors to cancer stem cells that contribute to tumorigenesis in a variety of tissues. In addition to laying the groundwork for investigating how the process of aging can affect tissue homeostasis, these studies also have important implications for stem cell-based therapies. We predict that tissue stem cells transplanted into older individuals may be unable to initiate self-renewing divisions to functionally replace damaged or diseased tissues without cotransplantation of “younger” niche cells or mobilization of endogenous stem cells from functional niches.

## EXPERIMENTAL PROCEDURES

### Fly Husbandry and Stocks

Flies were raised at 25°C on standard cornmeal-molasses-agar medium. Flies for aging experiments were supplemented with fresh yeast paste, and vials were changed every 3 days. Aging flies were obtained by collecting newly enclosed 0- to 1-day-old flies (~20 males and 20 females/vial, and ~30 males/vial when maintained in isolation). The *upd*GAL4, *UAS-gfp* (or *E132GAL4*, *UAS-gfp*) was a gift from E. Bach. Two *UAS-upd* lines were used (gifts from D. Harrison; Harrison et al., 1998): one transgene is inserted on chromosome II; the other

(*w<sup>+</sup>*; *UAS-upd*, TM2) is an insertion on chromosome III and consistently gives a stronger phenotype when overexpressed in early germ cells (Kiger et al., 2001; M.B. and D.L.J., unpublished data).

### Immunofluorescence

Testes were dissected into phosphate buffered saline (PBS) and examined using phase contrast microscopy. Immunofluorescence (IF) experiments on squashed testes were performed as described (Kiger et al., 2001) or as follows: testes were dissected and fixed in 2% PFA in PLP buffer (0.075 M lysine, 0.01 M sodium phosphate buffer pH7.4) for 1 hr at RT, rinsed in PBS, followed by standard IF staining, using antibodies listed in Supplemental Data.

### Quantification of GSCs and Hub Cells

Germ cells were counted as stem cells only when contacting FasIII<sup>+</sup> hub cells. Only those samples with an easily distinguishable hub of normal size (10–15 μm) were included in experiments to quantify GSC number. Hub cells were identified by analyzing serial sections through the tips of testes stained with an antibody to DN-cad and DAPI to stain hub cell nuclei (see Supplemental Data).

### BrdU Incorporation

BrdU incorporation was performed as described (Gönczy and DiNardo, 1996) with the following modifications: males were starved in empty vials with a wet vial plug inserted inside the vial overnight (14–18 hr). Flies were transferred without CO<sub>2</sub> into new vials and fed 100 mM BrdU in grape juice for 30 min and dissected immediately.

BrdU incorporation was analyzed using IF as follows: primary antibodies, other than anti-BrdU, were applied for 3 hr at 37°C or overnight at 4°C and fixed for 7 min in 4% formaldehyde. DNA was subsequently denatured by pretreating slides with 2 M HCl for 30 min. Samples were washed for 1 hr with PBS before the BrdU antibody was applied, and standard IF was performed.

### RNA In Situ Hybridization

RNA in situ hybridization was performed as described (Kiger et al., 2001). Probes were generated from linearized plasmid using the Roche Molecular Biochemicals RNA-labeling kit.

### Statistical Analyses

Statistical analyses were conducted using a Student's two-tailed t test assuming equal variance. Alpha values are significant when  $p < 0.05$ . Comparison of S phase indices was performed as described in the Supplemental Data.

### Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and four supplemental tables and can be found with this article online at <http://www.cellstemcell.com/cgi/content/full/1/4/470/DC1/>.

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